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Licenciada em Bioquímica



**The involvement of *CDH1* in Cancer angiogenesis**

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## **The involvement of *CDH1* in Cancer angiogenesis**

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## Abstract

Cancer is one of the leading causes of death worldwide. Biological hallmarks such as induced and sustained angiogenesis are implicated in tumour progression, as well as invasion and metastasis which are the major causes of cancer-related mortality. E-Cadherin impairment on the cell membrane is intimately related with invasion and metastasis. Also, increased levels of vascular endothelial growth factor (VEGF), an angiogenic marker, and its receptor on the plasma membrane can be implicated in tumour progression.

This work was focused on how the inactivation of E-Cadherin, a molecule associated to an invasive phenotype can be related with angiogenesis, probably through VEGF-A expression.

Two different cell lines without expression of E-Cadherin and stably transduced to express wild-type (WT) E-Cadherin were used to carry out this study: AGS Par/WT (from stomach) and MDA-435 Mock/WT (from breast). Immunohistochemical staining was performed to determine the cellular localization and western blot analysis was performed to assess the expression levels of E-Cadherin. *VEGFA* mRNA levels were assessed by quantitative Real-time PCR. Additionally, we determined the levels phosphorylated (phospho) ERK1/2, as well as the expression levels of total ERK1/2. To study the angiogenic role of E-cadherin the chick embryo Chorioallantoic Membrane (CAM) assay was used. We characterise *in vivo* the different cell lines concerning both angiogenic and tumorigenic responses dependent on E-Cadherin.

Only cell lines stably expressing WT human E-Cadherin showed levels of expression of this protein at the cell membrane regardless of their tissue of origin. *In vitro*, AGS and MDA-435 cells expressing WT E-Cadherin revealed an increased expression of *VEGFA* in comparison to the control although not statically significant. In addition, both phospho-ERK1/2 and total ERK1/2 presented similar levels of expression regardless of the tissue of origin and E-Cadherin expression. Both angiogenic and tumorigenic responses in AGS WT was significantly increased in comparison to the control. The MDA-435 WT cells revealed increased tumorigenic response in comparison to the control. Overall, these results suggest that E-Cadherin expression is important for micro-tumour formation as well as for neovascularisation but this effect is dependent on the *in vivo* context.

**Key words:** Cancer; E-Cadherin; (Tumour-) Angiogenesis; VEGF-A





## Resumo

O cancro ainda é uma das principais causas de morte no mundo. Processos biológicos como a angiogénese e a invasão ou as metástases, associados à progressão tumoral, são a maior causa de morte relacionada com esta doença. A diminuição de Caderina-E na membrana celular está directamente relacionada com o processo de invasão celular. Do mesmo modo, o aumento dos níveis do factor de crescimento vascular endotelial (VEGF, em inglês), um marcador angiogénico, e do seu receptor na membrana plasmática podem implicar um estado avançado da doença.

O objectivo do trabalho foi tentar compreender como é que a Caderina-E, uma molécula associada a um fenótipo invasivo pode estar relacionada com a angiogénese, provavelmente através da produção de VEGF-A.

Duas linhas celulares diferentes sem expressão de Caderina-E e estavelmente transfectadas para expressar Caderina-E “wild-type” (WT) foram utilizadas para realizar este estudo: AGS Par / WT (de estômago) e MDA-435 Mock / WT (de mama). As técnicas de Imunohistoquímica e Western Blot foram utilizadas para determinar a localização celular e a expressão proteica de Caderina-E, respectivamente. Os níveis de mRNA do *VEGFA* foram determinados por Real-time PCR. Os perfis de expressão de ERK1/2 fosforilada e total foram avaliados por Western Blot. O estudo do papel angiogénico da Caderina-E foi avaliado pelo ensaio da membrana corioalantóide (CAM, em inglês) de embrião de galinha. Caracterizou-se *in vivo* as diferentes linhas celulares no que a respeito às respostas angiogénica e tumorigénica dependentes da Caderina-E.

As linhas celulares que expressam Caderina-E WT apresentaram níveis de expressão da proteína na membrana celular, independentemente do tecido de origem. A coloração por imunohistoquímica revelou marcação positiva nas mesmas linhas celulares. As células AGS e MDA-435 que expressam Caderina-E apresentam um aumento não-significativo de expressão de *VEGFA*. O perfil de expressão das proteínas ERK1/2 apresentou níveis semelhantes nas linhas celulares, independentemente do tecido de origem e de expressão de Caderina-E. As células AGS WT apresentaram um aumento em ambas as respostas angiogénica e tumorigénica. As células MDA-435 WT mostraram um aumento na resposta tumorigénica em comparação ao controlo. Em conclusão, estes resultados sugerem que a Caderina-E é importante na formação de micro-tumores e na neovascularização, mas o seu efeito está dependente do contexto *in vivo*.

**Palavras-chave:** Cancro; Caderina-E; Angiogénese (tumoral); VEGF-A



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## **Abbreviations and Symbols List**

AGS – Human stomach adenocarcinoma (cell line)  
CAM – Chorioallantoic membrane  
CDH1 – Cadherin 1, type 1, E-Cadherin (epithelial)  
CIN – Chromosome instability  
DNA – Deoxyribonucleic acid  
EC – Cadherin domain  
EMT – Epithelial to mesenchymal transition  
ERK1/2 – Extracellular signal-regulated kinases 1 and 2  
IARC – International Agency for Research on Cancer  
LOH – loss of heterozygosity  
MAPK – Mitogen-activated protein kinase  
MDA (-435) – Human breast adenocarcinoma (-435) (cell line)  
MSI – Microsatellite instability  
o/n – over night  
(qReal-time) PCR – (quantitative Real-time) Polymerase chain reaction  
(m)RNA – (messenger) ribonucleic acid  
VEGF (-A) – Vascular endothelial growth factor (-A)  
VEGFR-2 – VEGF receptor 2  
WB – Western Blot  
WHO – World Health Organization  
WT – Wild-type







# Introduction

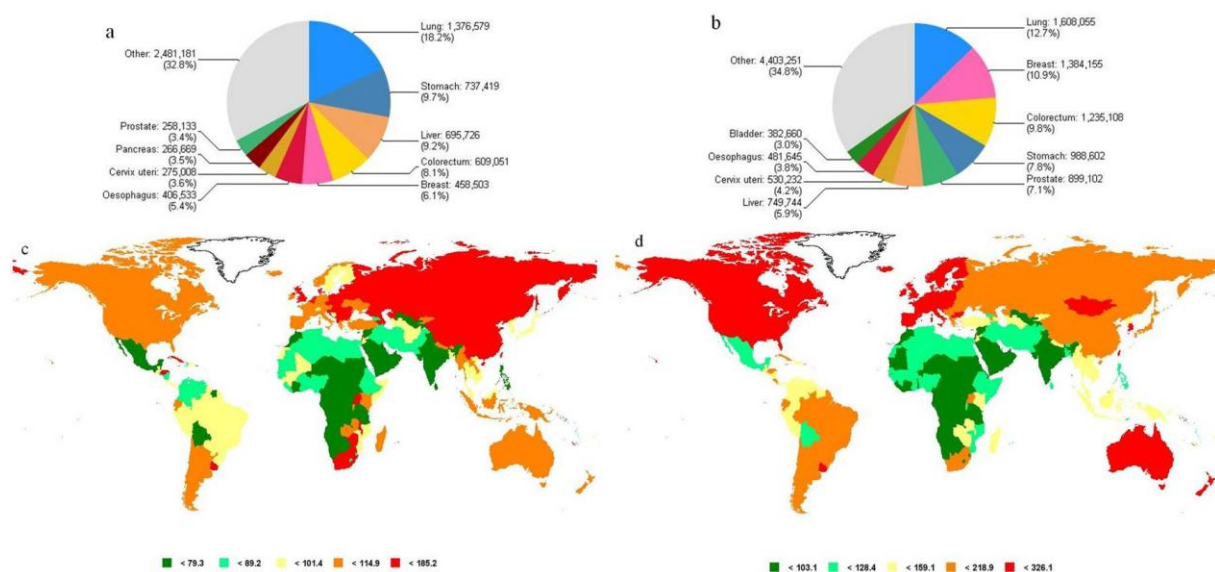
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## 1. Introduction

### 1.1. Cancer

According to the World Health Organization (WHO), cancer is a leading cause of death worldwide accounting for about 7.6 million deaths in 2008, which correspond to 13% of all deaths. Remarkable differences can be found in the incidence and death rates of specific forms of cancer around the world (Figures 1a and 1b; IARC, GLOBOCAN 2008). For example, lung, stomach and liver cancer are the top three leading types of cancer associated mortality (18.2%, 9.7% and 9.2%, respectively) (Figure 1a; IARC, GLOBOCAN 2008). The same is not observed when analysing cancer incidence: lung (12.7%), breast (10.9%) and colorectal cancer (9.8%) (Figure 1b; IARC, GLOBOCAN 2008).



**Figure 1 – Worldwide types of cancer mortality (a) and incidence (b) and world distribution of cancer mortality (c) and incidence (d) (age-standardised rates/100,000population) in year 2008 for both sexes and all ages (IARC, GLOBOCAN 2008).**

In addition, other factors can be associated with cancer risk differences, such as environment and culture (Figures 1c and 1d; IARC, GLOBOCAN 2008). Geographical variation studies of migrant populations have shown that subsequent generations of migrant individuals appear to acquire gradually the risk levels of the host country.

Although, insights into the possible causes of cancer can be obtained by epidemiologic studies that relate environment and racial and cultural influences to the occurrence of malignant neoplasms, cancer is a disorder of cell invasion and in the majority of cases also of cell growth and ultimately, the causes should be defined at the tissue, cellular and subcellular levels (Hanahan and Weinberg 2000).

Cancer, the common designation for malignant tumour is characterized by the uncontrolled ability of cells to cross and invade physical barriers. These cells are able to invade the surrounding tissue and frequently metastasize to distant organs, which is the major cause of death from cancer (van Zijl,

Krupitza et al. 2011) All types of cancer share similar characteristics. Normal mammalian cells have molecular mechanisms regulating their growth, differentiation, and death. Once a group of cells escape these normal regulatory mechanisms, progressively acquire the ability to infiltrate and invade locally. Hereby, cells have the opportunity to spread and generate metastasis, marking unequivocally a tumour as malignant (Hanahan and Weinberg 2000).

Cancer initiation is a multi-step process where cells accumulate a series of genetic alterations (Bertram 2000; Hanahan and Weinberg 2000). Nonlethal genetic alterations or mutations are the main cause for cancer initiation, leading to a process of transformation. Such mutations may be acquired by the action of environmental agents or be inherited in the germline (Cornelisse and Devilee 1997). Genetic instability of cancer cells seems to be the more accurate system to explain cancer progression. Genomic instability is broadly classified into microsatellite instability (MSI) and chromosome instability (CIN) (Charames and Bapat 2003). For this matter, microsatellite instability (MSI) involves small mutations in microsatellites regions (short sequences of a few base pairs in length that are repeated multiple times). The insufficiency of DNA mismatch repair system during DNA replication is the main cause for the increased rate of mutations at the nucleotide level. Also, chromosomal instability (CIN) may be responsible for the insufficiency in chromosome segregation checkpoints, due to the chromosomal gains or losses (Jallepalli and Lengauer 2001).

There are two main classes of regulatory genes that appear to be targets for genetic alterations, tumour suppressor genes and oncogenes (Cornelisse and Devilee 1997; Bertram 2000). Changes in both these genes can trigger cancer, either by the silencing of tumour protective genes or the activation of tumour promoting genes, respectively.

Oncogenes derive from proto-oncogenes. Structural alterations or changes in the regulation of gene expression, can transform proto-oncogenes into oncogenes. The main difference between them is that proto-oncogenes promote normal growth and differentiation, whereas oncogenes once activated synthesize abnormal gene product or enhance the production of the normal protein (Cornelisse and Devilee 1997; Rak and Yu 2004).

On the contrary, tumour suppressor genes are crucial for inhibition of cell division, as well as survival or other properties of tumour cells. Therefore, both alleles must be damaged for transformation to occur. The main causes for tumour suppressor genes inactivation are point mutations, deletions or insertions in sites that are essential for expression or function, as well as epigenetic silencing by promoter methylation or overexpression of transcription repressors (Yeo 1999; Sherr 2004).

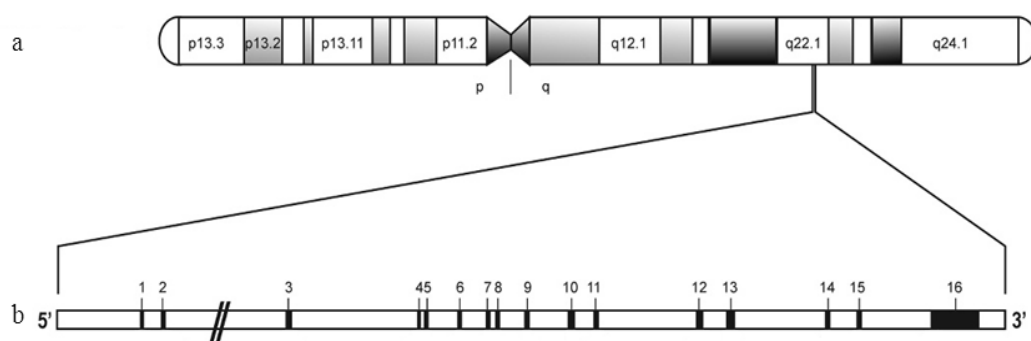
Alterations in these two sets of genes may lead, for example, to increased cell division and aberrant differentiation. Hanahan and Weinberg have recently reviewed that the organizing principles of malignant tumours have undergone a conceptual progress in the last decade (Hanahan and Weinberg



2000; Hanahan and Weinberg 2011). The classical hallmarks of cancer are: 1) resistance to cell death; 2) self-sufficiency in growth signals; 3) insensitivity to growth inhibitory signals; 4) limitless replicative potential; 5) sustained angiogenesis; 6) tissue invasion and metastasis. More recently, deregulation of cellular energetics, evasion from immune destruction, genome instability and mutation as well as tumour-promoting inflammation, have been added as novel cancer hallmarks. Although, all the hallmarks are responsible for the events occurring in carcinogenesis and dictate tumour growth, which depend on the imbalance between cell production and cell loss, induced and sustained angiogenesis is a biological hallmark for malignant progression. Here, blood supply is crucial to tumour maintenance and growth, which cannot enlarge beyond 1 to 2 mm in diameter unless it is vascularised. Also, invasion and metastasis are biological hallmarks of malignant tumours and the major cause of cancer-related mortality.

## 1.2. E-Cadherin

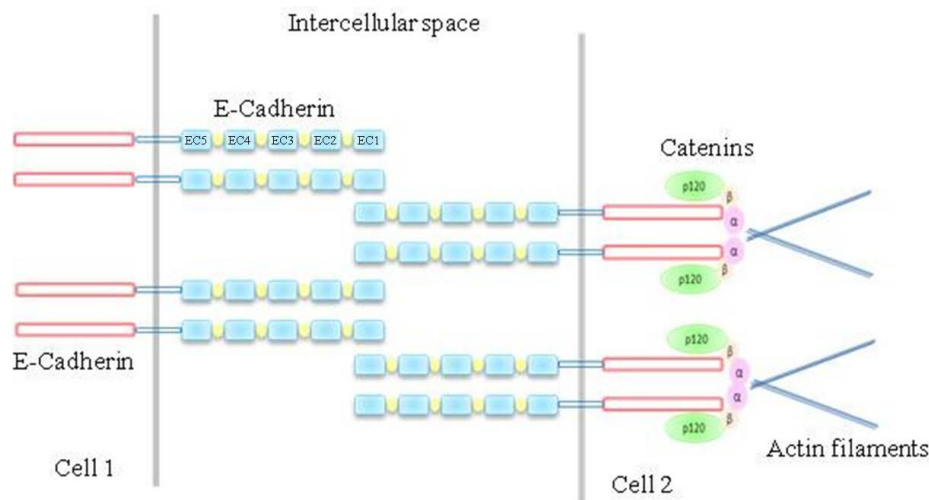
The human E-Cadherin is encoded by the *CDH1* gene located in the long arm of chromosome 16, 16q22.1 (Figure 2). The gene comprises 16 exons and 15 introns and the coding region is translated into a 120kDa protein (www.ensembl.org). The intron-exon boundaries are highly conserved and the 5' CpG island is highly-dense, where introns 1 and 2 appear to have a regulatory role in addition to the promoter region and the latter is unusually large (Berx, Staes et al. 1995).



**Figure 2 – Location within the long arm of the chromosome 16, 16q22.1 (a) of the *CDH1* gene (b) (adapted from (van Roy and Berx 2008)).**

E-Cadherin is a member of the superfamily of Cadherins, a family of adhesion molecules characterized by a  $\text{Ca}^{2+}$ -dependent cell-cell adhesion (Hulpiau and van Roy 2009). The Cadherins family are organized comprising three main domains: a short cytoplasmic domain, a single transmembrane segment, and a large extracellular domain with five Cadherin motifs or EC domains. These domains are characterized by being tandemly repeated domains responsible for their cell-cell adhesive function (van Roy and Berx 2008). The first to be identified and most studied was the “classical” Cadherins is E-Cadherin, due to its wide expression in all epithelial tissues (Hulpiau and

van Roy 2009). Also, its role in the different regulatory processes have been a reason for further studies (Takeichi 1995; Cavallaro and Christofori 2004).



**Figure 3 - Human E-Cadherin structure and binding profile scheme showing the interaction with p120 catenin (p120), β-catenin (β) and α-catenin (α) complex bound to actin filaments (based on Yilmaz and Christofori 2010).**

The EC domains have  $\text{Ca}^{2+}$ -binding sites necessary for E-Cadherin stabilization in a way that cell-cell adhesion mediated by E-Cadherin only occurs in a  $\text{Ca}^{2+}$ -dependent fashion. The cytoplasmic domain of E-Cadherin is associated with catenins that bridge the linkage with actin filaments within the cytoskeleton (Takeichi 1995). This association with catenins is essential to provide proper cell-cell adhesion. Both functional extracellular and cytoplasmic domains work together to modulate stable cell-cell contacts (Berx, Staes et al. 1995). The recognition and binding between neighbouring cells is done in a homophilic fashion by the EC1 and EC2 fragments of E-Cadherin maintained by  $\text{Ca}^{2+}$ . Moreover, the cytoplasmic domain is responsible to form the complex with the cytoskeleton by association between catenins and actin filaments (Figure 3) (Yap, Brieher et al. 1997).

E-Cadherin is one of the proteins specialized in the adhesive function of cells and works together dynamically with a variety of transmembrane glycoproteins by influencing their signalling properties (Ozawa and Kemler 1990; Takeichi 1995).

E-Cadherin has also a pivotal role during embryonic development being expressed in early stages, at two-cell stage, allowing cell migration and morphogenesis. E-Cadherin is also involved in the maintenance and in the homeostasis of adult epithelial tissue integrity and structure providing the landmarks for cells confining them spatially (Larue, Ohsugi et al. 1994).

A dynamic process such as Cadherin-mediated adhesion is regulated by several signal transduction pathways. There is also evidence that Cadherins are not only targets for signalling pathways that regulate adhesion, but may themselves send signals that regulate basic cellular processes, such as

migration, proliferation apoptosis and cell differentiation (Guilford 1999; Cavallaro and Christofori 2001; van Roy and Berx 2008).

### **1.2.1. *CDH1/E-Cadherin: a Tumour/Invasion Suppressor Gene***

One of the pivotal mechanisms responsible for tumour initiation and progression is the control of cellular adhesion and motility (Guilford 1999). Here, E-Cadherin plays a major role in malignant cell transformation and especially in tumour development and progression (Takeichi 1993; Guilford 1999).

E-Cadherin exhibits an important role during the epithelial-mesenchymal transition (EMT), which is a process of cell conversion that occurs during development. This process is thought to be occurring in an uncontrolled fashion in malignant tumours of epithelial origin (Thiery 2002). Malignant carcinoma cells are characterized in general by poor intercellular adhesion, loss of the differentiated epithelial morphology and increased cellular motility. These functional features are frequently associated with downregulation or a complete abrogation of E-Cadherin expression, mutation(s) of the E-Cadherin gene, or other mechanisms interfering with the integrity of adherent junctions (Riethmacher, Brinkmann et al. 1995). In human tumours, the loss of E-Cadherin-mediated cell adhesion is one rate-limiting step correlated with the loss of epithelial morphology and with the acquisition of metastatic potential by the carcinoma cells. Thus, a direct role in the suppression of tumour invasion has been assigned to E-Cadherin (Knudson, Strong et al. 1973; Berx, Cleton-Jansen et al. 1995; Guilford 1999).

The reduction of E-Cadherin expression is considered one of the most important events involved in dysfunction of cell-cell adhesion system (Vleminckx, Vakaet et al. 1991).

Multiple mechanisms are found underlying the inactivation of E-Cadherin-mediated cell adhesion during tumorigenesis (Guilford 1999; Cavallaro, Schaffhauser et al. 2002). The cyclical regulation of E-Cadherin expression by transcriptional and post-translational mechanisms provides a complex array of mechanisms leading to E-Cadherin downregulation (Figure 4) . These reflect the cell's need to balance proliferation with adhesion and motility (Guilford 1999). On the other hand, an irreversible mutation on the *CDH1* gene provides a simple mechanism for E-Cadherin-mediated cell adhesion inactivation (Figure 4; Berx, Cleton-Jansen et al. 1995; Guilford 1999; Cavallaro, Schaffhauser et al. 2002).

*CDH1* gene is located in a region frequently showing loss of heterozygosity (LOH) within the 16q chromosome, in many types of human carcinomas (Figure 4; Berx, Staes et al. 1995). Also, several inactivating mutations in the coding region are often observed in sporadic lobular breast cancer and diffuse gastric cancer. Frameshift and nonsense are frequently associated with breast cancer, rather than the missense mutations and in-frame deletions predominant in gastric cancers (Berox, Cleton-Jansen et al. 1995; Guilford 1999; Oliveira, Seruca et al. 2006). Germline mutations can also occur and are described as the cause of Hereditary Diffuse Gastric Cancer (HDGC) syndrome (Caldas,

Carneiro et al. 1999; Oliveira, Seruca et al. 2006). Beyond that, E-cadherin expression can also be regulated at the promoter level by methylation, as well as by the direct binding of specific transcriptional factors (Machado, Oliveira et al. 2001).

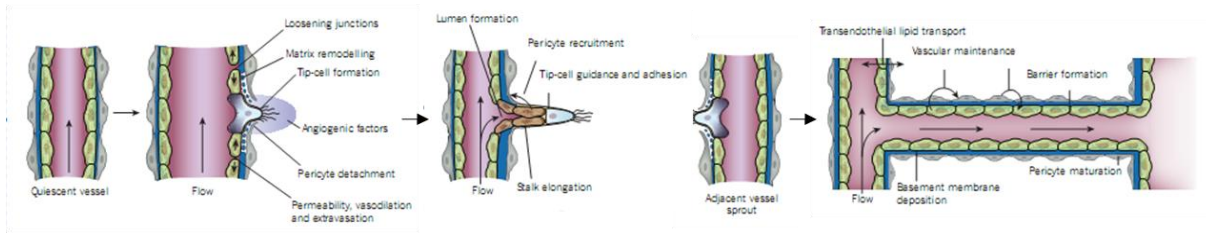
### **1.3. Angiogenesis**

Blood vessels are crucial to carry nutrients, oxygen and hormones to distant organs. The physiologic process involving the development of new blood vessels from pre-existing vascular structures is designated Angiogenesis (Carmeliet and Jain 2011).

Vascular endothelial cells are the main elements of blood vessels. These cells have autocrine actions mediated through several molecules, being the most relevant the vascular endothelial growth factor (VEGF). These cells are interconnected by junctional molecules, such as vascular endothelial (VE)-Cadherin, and are ensheathed by pericytes (Liekens, De Clercq et al. 2001; Carmeliet and Jain 2011).

The VEGF family has few members that include VEGF-A, -B, -C, -D, -E and placenta growth factor (PlGF) (Ferrara 2009). VEGF-A is the main component of the VEGF family and it regulates angiogenesis mainly through activation of VEGF receptor-2 (VEGFR-2) present in endothelial cells. VEGF-A effects include vessel enlargement, increased vessel branching and maintenance of vascular homeostasis (Folkman and Shing 1992). When VEGF-A is lost, failure of vascular development occurs (Folkman, Watson et al. 1989; McMahon 2000; Carmeliet and Jain 2011).

The molecular basis of vessel branching (Figure 4) involves the stimulation with angiogenic factors, which allows the quiescent vessel to dilate and an endothelial cell is selected, the tip-cell. The degradation of the basement membrane is a requirement for tip-cell formation, as well as pericyte detachment and loosening of endothelial cell junctions. The increased permeability permits the extravasation of plasma proteins to deposit a provisional matrix layer, and proteases remodel pre-existing interstitial matrix, all enabling cell migration. In response to external guidance signals tip-cells migrate and stalk cells behind the tip-cell proliferate, elongate, form a lumen and sprout to establish a perfused neovessel. After fusion of neighbouring branches, lumen formation allows perfusion of the neovessel, which resumes quiescence by the re-establishment of junctions, deposition of basement membrane, maturation of pericytes and production of vascular maintenance signals (McMahon 2000; Carmeliet 2003; Hofer and Schweighofer 2007).



**Figure 4 - The molecular basis of vessel branching (adapted from (Carmeliet and Jain 2011)).**

New and pre-existing blood vessels are essential for tissue growth and regeneration due to the ability of adult endothelial cells to rapidly grow and sprout in response to physiological stimuli. However, blood vessels also play an important role in disease, particularly in inflammation and in tumour progression.

### **1.3.1. Tumour Angiogenesis**

Tumour cells have a constant need for oxygen and nutrient supply which they achieve through new blood vessels, allowing their growth (Ross 1989). Thus, tumour vascularisation is a pivotal process for malignancy progression (Carmeliet and Jain 2000; Hanahan and Weinberg 2000; McMahon 2000; Carmeliet 2003; Hanahan and Weinberg 2011).

The angiogenic switch and the vascular phase are the two main phases of the angiogenic cascade leading to tumour vascularisation (Bergers and Benjamin 2003). The angiogenic switch is characterised by the transition from a dormant non-vascular tumour cells mass to a highly vascularised and progressively outgrowing tumour (Hanahan and Folkman 1996). It is a time-restricted event during tumour progression where the balance between pro- and anti-angiogenic factors deflects towards a pro-angiogenic outcome (Folkman, Watson et al. 1989; Baeriswyl and Christofori 2009). During tumour progression, the angiogenic switch depends on both stimulatory and inhibitory molecules produced by tumour cells and on cell-surface receptors displayed by the vascular endothelial cells (Carmeliet and Jain 2000; Carmeliet and Jain 2011). Moreover, the newly tumour neovasculature is characterized by precocious capillary sprouting, excessive vessel branching, enlarged vessels and leakiness (McMahon 2000; Cavallaro, Liebner et al. 2006).

Pericytes play an important role in normal tissue vasculature as mentioned, but pericytes coverage is also crucial to maintain a functional neovasculature within a tumour avoiding necrosis and tumour cell apoptosis (Folkman, Watson et al. 1989; Folkman and Shing 1992). Endothelial cells closely opposed to outer surfaces need pericytes coverage for mechanical and physiological support (Raza, Franklin et al. 2010). Moreover, tumour-associated vasculature have pericytes associated with the endothelial cells, even though in a loosely fashion (Folkman, Watson et al. 1989; Baeriswyl and Christofori 2009; Raza, Franklin et al. 2010).

### **1.3.2. Assays to evaluate Angiogenesis**

Endothelial cell migration, proliferation, differentiation and structural rearrangement are essential to the angiogenic process. However, other cell types are involved in tumour angiogenesis including tumour cells themselves and circulating blood cells (Auerbach, Auerbach et al. 1991; Staton, Stribbling et al. 2004). Currently, it is not possible to model or simulate this complex process *in vitro*. *In vitro* models to assess angiogenesis are focused predominantly on migration, proliferation and tubule formation by endothelial cells upon stimulatory or inhibitory response. Therefore, the comparison between different *in vitro* systems is difficult (Auerbach, Akhtar et al. 2000). *In vivo* assays are essential to evaluate angiogenesis in conditions that reproduce more accurately tumour induced angiogenesis in humans. *In vivo* assays do not show limitations regarding the origin of endothelial cells or the composition of the media used for culture (Staton, Stribbling et al. 2004). However, limitations also exist depending on the specificity of the microenvironment and the species used (Auerbach, Akhtar et al. 2000; Auerbach, Lewis et al. 2003).

#### 1.3.2.1. Chick Embryos and the Chorioallantoic Membrane (CAM) assay

*In vivo* angiogenic assays should be reliable, technically straightforward, easily quantifiable and, most importantly, physiologically relevant. Nevertheless, *in vivo* angiogenesis assays may also be sensitive to environmental alterations (Ribatti, Vacca et al. 1996; Staton, Stribbling et al. 2004). The chick CAM is one of the most widely used *in vivo* assay for studying angiogenesis and its regulators (Hasan, Shnyder et al. 2004; Goodwin 2007).

The CAM is an extraembryonic membrane constituted by an ectodermal chorionic epithelium, an intermediate mesoderm and an endodermal allantoic epithelium (Figure 5) (Gabrielli and Accili 2010). The CAM development starts on day 4 of incubation by fusion of the chorion and the allantois. Immature blood vessels scattered in the mesoderm grow exponentially until day 8/9 and give rise to a capillary plexus, which mediates gas exchange with the outer environment. The capillary proliferation continues and from day 14 forward, the endothelial cell mitotic index declines rapidly, and the vascular system reaches its final arrangement on day 18 (Ribatti, Vacca et al. 1996; Gabrielli and Accili 2010).



**Figure 5 – Schematic representation of the three tissue layers of the chick CAM: the outer shell membrane (sm) where the chorionic epithelium (ce) is attached; the intermediate mesenchymal area (m); and the allantoic epithelium (ae) (adapted from (Gabrielli and Accili 2010)).**

Cells and/or molecules can be inoculated into the CAM directly or with a variety of carriers and

physical supports. The test material can be introduced in small pieces of polymerized materials, such as biologically inert synthetic polymers or within small rings limiting the inoculation site. The formation of new blood vessels can afterwards be analysed in terms of number, diameter, density, permeability, branch point number and blood flow (Ribatti, Vacca et al. 1996; Hasan, Shnyder et al. 2004; Gabrielli and Accili 2010).

One disadvantage of CAM assay is that the CAM already contains a well-developed vascular network and the vessel permeability can affect the assessment of the effects from the test substance. Another limitation is that the assay is performed in an embryo and as a model may be difficult to mimic adult tissues responses and processes during tumour progression. Also the fact that the chick embryo is not a mammal can represent a disadvantage (Auerbach, Lewis et al. 2003; Hasan, Shnyder et al. 2004).

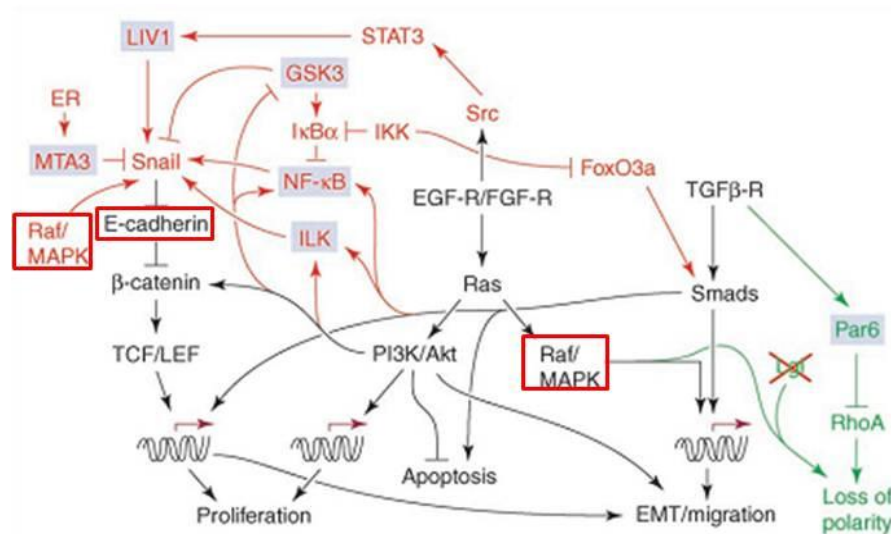
#### **1.4. E-Cadherin association with Angiogenesis**

As described in the literature, E-Cadherin and VEGF are important molecules involved in tumour metastasis (Gupta and Massague 2006; Oppenheimer 2006; Yilmaz and Christofori 2010; van Zijl, Krupitza et al. 2011). Studies revealed a clinic-pathological evidence that reduced expression of E-Cadherin was correlated with poor tumour differentiation and deeper tumour invasion, along with an increased expression of VEGF-A (Bazas, Lukyanova et al. 2008; Zhou, Li et al. 2010). Abnormal expressions of E-Cadherin and VEGF are frequent features in cancer tissues and may represent one of the early molecular changes in the development of epithelial cancer (Takeichi 1993; Bertram 2000; Gupta and Massague 2006; Kroemer and Pouyssegur 2008; Ferrara 2009). However, there is no direct relationship on the prognostic value of expression level of the components of cell-to-cell adhesion system and the markers of angiogenesis in cancer.

The relation between expression of cell-to-cell adhesion molecules, i.e. E-Cadherin, and VEGF and clinic-pathological characteristics of tumours and survival time revealed exists (Bazas, Lukyanova et al. 2008; Zhou, Li et al. 2010). However, it is not clear that these molecules interact with each other. The presence of E-Cadherin in tumours correlates with the absence of metastases. While the level of VEGF expression correlates with the degree of injury (Bazas, Lukyanova et al. 2008). Along with this, the presence of E-Cadherin is associated with favourable prognosis in most types of cancer (Inoue, Kamada et al. 2002; Reddy, Liu et al. 2005). On the contrary, VEGF is a marker of unfavourable disease course and its expression is characteristic for late stages of the disease and shorter survival (McMahon 2000; Bazas, Lukyanova et al. 2008; Kroemer and Pouyssegur 2008).

Signalling pathways have been assigned to interact with both E-Cadherin and VEGF-A (Pece and Gutkind 2000; Kumar, Shen et al. 2009).

E-cadherin may regulate the activity of MAPK, a key signalling pathway involved in cell fate decisions, upon the formation of cell-cell contacts among neighbouring cells (Reddy, Liu et al. 2005). However, it is known that MAPK signalling pathway also interplay with adherent junction molecules, as E-Cadherin, by inducing the expression of its transcriptional repressors (Figure 6) (Pece and Gutkind 2000). Together, these findings suggest a crucial role of E-Cadherin in transducing signals outside-in through the engagement of tyrosine kinase receptors and provide an attractive mechanism whereby these cell-adhesion molecules can affect cell fate decision upon cell-cell contact formation (Figure6; Huber et al. 2005; Qian, Karpova et al. 2004).



**Figure 6 – Scheme suggesting the interplay between MAPK signalling and E-Cadherin regulation (adapted from (Huber et al. 2005)).**

Furthermore, MAPK signalling pathway is also associated with VEGF-A signalling pathway, leading to cell proliferation, and apparently both have a stimulatory role within one another (Sekulic, Haluska et al. 2008; Kumar, Shen et al. 2009).

All tissues and cell types virtually express VEGF mRNA and is particularly elevated in highly vascularised tissue and in tumour-derived cell lines (Berra, Pages et al. 2000; Pages, Milanini et al. 2000). Studies have suggested a role for ERK1/2 in VEGF-induced hyperpermeability (Pages, Milanini et al. 2000; Breslin, Pappas et al. 2003). Growth factors are thought to activate ERK-1/2 not only through the Ras-Raf-MEK pathway but also through p38 and PI3K (Figure 7) (Varma, Breslin et al. 2002; Kumar, Shen et al. 2009; Walczak, Gaignier et al. 2011; Guo et al. 2010). Also, during cell culture, deprivation of oxygen mimics the necrotic hypoxic regions in solid tumours induces VEGF mRNA expression by both an increase in the rate of transcription but also by stabilization of its mRNA (Berra, Milanini et al. 2000; Berra, Pages et al. 2000; Pages, Milanini et al. 2000). MAP kinase signalling, a module for transducing cell surface signals to the nucleus controls cell-cycle entries, at



the level of vascular endothelial cells (Figure 7) (Berra, Milanini et al. 2000; Berra, Pages et al. 2000; Pages, Milanini et al. 2000; Ferrara 2009; Guo et al. 2010).

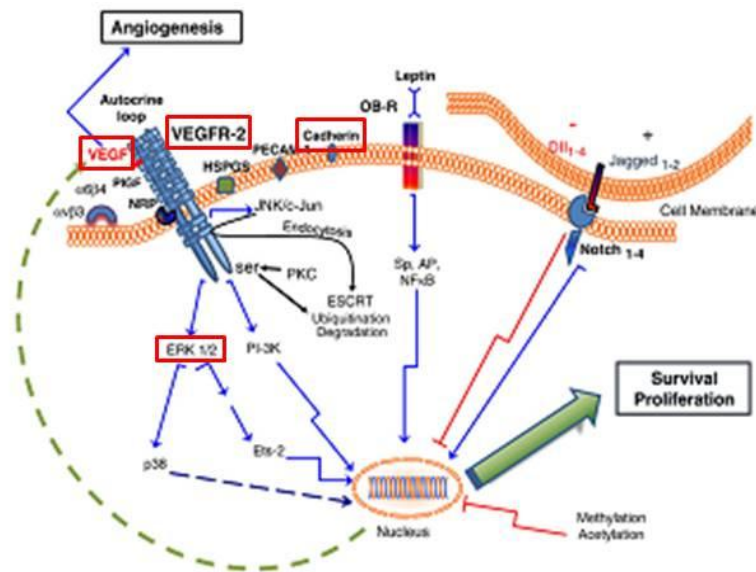


Figure 7 – Scheme suggesting the interplay between MAPK and VEGF-A signalling pathways and co-regulation through VEGF-A signalling pathway and/or through MAP kinase (adapted from (Guo et al. 2010)).



## Project Aims

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## 2. Project Aims

Cancer is still one of the leading causes of death worldwide. Biological hallmarks such as induced and sustained angiogenesis are implicated in tumour progression, as well as invasion and metastasis which are the major causes of cancer-related mortality. E-Cadherin impairment on the cell membrane is associated to tumour progression. Also, increased levels of VEGF, an angiogenic marker, and its receptor on the plasma membrane can be implicated during tumour development.

There is an association between expression of cell-to-cell adhesion molecules, i.e. E-Cadherin, and VEGF and clinic-pathological characteristics of epithelial tumours and survival time (Bazas, Lukyanova et al. 2008; Zhou, Li et al. 2010). However, it is not clear that these molecules interact with each other.

To determine whether E-Cadherin expression can be related with tumour angiogenesis it was proposed to:

1. Characterize different epithelial cell lines, regarding *CDH1*/E-Cadherin, through:
  - a. mRNA transcription
  - b. protein expression
2. Determine E-Cadherin role in tumour angiogenesis *in vivo* by assessing both:
  - a. Angiogenic response
  - b. Tumorigenic response
3. Validate *in vivo* results through:
  - a. VEGF-A mRNA transcription
  - b. (phospho-)ERK1/2 expression
  - c. E-Cadherin cellular localization in CAM tumours



## Materials and Methods

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### **3. Materials and Methods**

#### **3.1. Cell Lines and Culture Conditions**

In this study two different epithelial cell lines were used: MDA-435 and AGS.

All used cell lines endogenously lack E-Cadherin expression at protein level. In our group, stably expressing wild-type E-Cadherin cell lines have been previously established (MDA-435 WT and AGS WT). Here, parental cell line (AGS Par) or cells transfected with the empty vector (MDA-435 Mock) were used as control in parallel with the respective WT E-Cadherin expressing cell lines.

AGS (Par) cells from human gastric cancer were grown in Roswell Park Memorial Institute (RPMI-1640) medium (Gibco, Invitrogen) supplemented with 10% foetal bovine serum (FBS; HyClone, Thermo Scientific) and 1% Penicillin-Streptomycin (PS; Gibco, Invitrogen). The AGS WT cells medium was supplemented with 5 µg/mL of Blasticidin (Gibco, Invitrogen).

The human cell line MDA-MB-435S (MDA-435), from breast cancer, was maintained in Dulbecco's Modified Eagle Medium (DMEM) medium (Gibco, Invitrogen) supplemented with 10% FBS and 1% PS. The medium for MDA-435 WT cells was additionally supplemented with 500 µg/mL of Geneticin (G418; Gibco, Invitrogen), and for MDA-435 Mock cells the medium was supplemented with 5 µg/mL of Blasticidin.

The cells were maintained in an incubator Heraeus at 37°C under a humidified atmosphere containing 5% CO<sub>2</sub>.

#### **3.2. Protein Extraction and Quantification**

Cells were plated in a 6-well culture dish and maintained under the same culture conditions previously described.

At approximately 80% of confluence, cell cultures were washed with phosphate buffered saline (PBS) solution and total cell lysates were obtained through lysis with 1% Triton X-100 (Sigma), 1% Nonidet P-40 (Sigma) in PBS supplemented with a protease inhibitor cocktail (Roche) and a phosphatase inhibitor cocktail (Sigma). Total protein lysates were obtained after centrifugation at 4°C for 20 minutes and the resulting pellets were discarded.

For total protein quantification, the modified Bradford proteins assay (Bio-Rad) was executed according to the protocol provided by the manufacture. To construct the standard curve, Bovine Serum Albumin (BSA; Sigma) was used in different concentrations ranging from 0,25mg/mL to 3mg/mL. Samples were analysed on a 96-well plate for an ELISA spectrophotometer (Bio-Rad) at 655nm.

### 3.3. SDS-PAGE and Western Blot

Equal amounts of total protein (25µg) from each cell lysates were subjected to a 10% SDS-PAGE electrophoresis buffered in Running Buffer after samples mixture with Laemmli sampling buffer 1x at 95°C for 5 minutes. Proteins were separated by molecular weight and according to their electrophoretic mobility under 120V for 1.5 hours.

Resolved proteins were transferred to a Hybond nitrocellulose membrane (Amersham, GE Healthcare) in a Western Blot Sandwich in a wet transfer system buffered with Transfer Buffer 1x under 100V for 1.5 hours.

Separated proteins within the nitrocellulose membrane were blocked with 5% non-fat dry milk and PBS-Tween 0.5% for at least 1 hour at room temperature (RT). Proteins were probed using different primary antibodies targeted for proteins of interest at 4°C overnight (o/n), and horseradish peroxidase (HRP) conjugated antibodies were used as secondary specific targeted antibodies under mild shake for 1 hour. Antibodies used in WB are listed in Table 1 as well as respective dilution and blocking conditions.

**Table 1 - Antibodies used in WB experiments, with respective dilution and blocking conditions.**

Antibody	Dilution and Blocking	Time/Temp
<b>Primary Antibodies</b>		
Mouse anti-E-Cadherin (120kDa; BD Biosciences)	1:3000 in 5% non-fat dry milk	o/n at 4°C
Rabbit anti-p42/44 MAPK (ERK 1/2) (42/44 kDa; Cell Signaling)	1:1000 in 4% BSA	o/n at 4°C
Rabbit anti-Phospho-p42/44 MAPK (Thr 202/Tyr 204) (42/44 kDa; Cell Signaling)	1:1000 in 4% BSA	o/n at 4°C
Goat anti-α-tubulin (55kDa; Sigma)	1:10000 in 5% non-fat dry milk	o/n at 4°C
<b>Secondary Antibodies</b>		
Goat anti-Mouse HRP conjugated (Amersham, GE Healthcare)	1:3000 in 5% non-fat dry milk	1h at RT
Goat anti-Rabbit HRP conjugated (Amersham, GE Healthcare)	1:3000 in 5% non-fat dry milk	1h at RT

Immunodetection was performed using an enhanced chemiluminescence, ECL Plus, kit (Amersham, GE Healthcare). The chemiluminescent substrate and the enhancer solution were mixed 1:1 proportion and blots were obtained by exposing the membranes to photographic films (Amersham, GE Healthcare) for increasing time periods ranging from 1 second up to 10 minutes according to the band strength.

When membrane reprobing was intended, the membranes were first washed with an isotonic solution for 20 minutes, incubated for 30 minutes at 50°C with a Stripping Solution (10% SDS, Tris-HCl 1M and  $\beta$ -mercaptoethanol) and washed with PBS1x for 20 minutes.

Immunoblots were quantified using Quantity One 1-D Analysis Software (Bio-Rad) and the background value was deduced to each protein band value. Final values correspond to normalized values to the loading control value,  $\alpha$ -tubulin, of the same gel.

### **3.4. RNA extraction and analysis of mRNA expression by RT-PCR and real-time PCR**

Total RNA was extracted from AGS Par, AGS WT, MDA-435 Mock and MDA-435 WT cells using TriPure Isolation Reagent (Roche).

Accordingly with the instruction manual, chloroform was added to the lysed cells to separate the solution in three phases. To the aqueous/upper phase was added isopropanol, after which the RNA pellet was obtained and washed with 75% ethanol. The excess ethanol from the RNA pellet was removed by air-drying or under vacuum and the RNA pellet was resuspended in Diethylpyrocarbonate (DEPC)-treated RNase-free water and dissolved at 55°C for 10 minutes. In each step of this procedure was performed a centrifugation at 14000rpm for 10 minutes at 4°C and the supernatant discarded, unless when were obtained the three phases separation in which the organic and the interphase were discarded.

Total RNA was quantified using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies) and DEPC-treated RNase-free water (DEPC-treated water) was used as negative control.

RNA was used to synthesise single-strand cDNA with Superscript II Reverse Transcriptase (Invitrogen). Approximately 1  $\mu$ g of total RNA was used in a mixture with 100ng of Random Primers (Invitrogen) and DEPC-treated water to 12  $\mu$ L of volume. The mixture was heated at 70°C for 10 minutes followed by cooling it on ice for 2 minutes. To each sample were added 4  $\mu$ L of 5x First-Strand Buffer (Invitrogen), 2  $\mu$ L of DTT (0.1 M; Invitrogen), 1  $\mu$ L of RNA dNTPs (Bioron) with 2.5mM each, 0,2  $\mu$ L RNAsin (2500U/ $\mu$ L; Promega), 0,75  $\mu$ L of Superscript II Reverse Transcriptase (Invitrogen) and 0,75  $\mu$ L of DEPC-treated water.

*VEGFA* and *CDH1* expression was assessed using the Real Time PCR technique and TaqMan Gene Expression Assays (Hs00900055\_m1 and Hs01023895\_m1, respectively; Applied Biosystems). For each sample, approximately 40ng of cDNA were used to a final reaction volume of 10  $\mu$ L PCR mix containing: 5  $\mu$ L of TaqMan Master Mix (Applied Biosystems), 1.6  $\mu$ L of probe and 3.5  $\mu$ L of DEPC-treated water. GAPDH (Hs99999905\_m1; Applied Biosystems) was used as endogenous control. The thermal profile of the run method included two holding stages at 50°C for 2 minutes and at 95°C for

10 minutes, followed by 42 cycles of 95°C for 15 seconds and 60°C for 1 minute. The resulting data was analysed using the 7500 Software For 7500 and 7500 Fast Real-time PCR Products (v2.0.4; Applied Biosystems).

### 3.5. Chicken Chorioallantoic Membrane (CAM) Assay

This *in vivo* assay was performed to assess both tumorigenic and angiogenic potential of the different cell lines.

Fertilized eggs were placed into an incubator (Termaks) with humidified atmosphere at 37.5°C (Embryonic Development Day 0; EDD0). The eggs were maintained horizontally and rotated several times a day to mimic natural incubation in early days of development.

Subsequent to incubation, at EDD3, a portion of eggshell was removed after removing 2 mL of albumen with a sterile syringe and a 26/28G needle. Thus, the air sack was displaced and the inner membrane was detached from the eggshell.

Prior to topic cell inoculation at EDD10, cells maintained under the conditions described previously were prepared through trypsin treatment, centrifugation at 1200rpm for 5 minutes and resuspended in serum free culture medium. Counted with a Neubauer chamber (DanLab), equal amounts of cells ( $1 \times 10^6$  cells/10 $\mu$ L/egg) from each cell type were inoculated onto the CAM, inside a silicon ring with 3mm in diameter.

At EDD14, the embryos were sacrificed by adding approximately 2 mL of 4% paraformaldehyde (PFA) fixative. After 1h, the silicon ring was carefully removed and the area of interest dissected and photographed in the stereomicroscope (OLYMPUS, SZX2-ILLT). Tissue samples were preserved in PFA 4% until further processing.

Figure 8 summarises the key time points of the CAM assay. Figure 9 illustrates the inoculation site (silicon ring) of an EDD14 chick embryo.

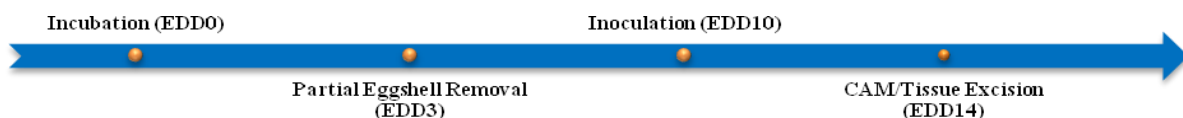
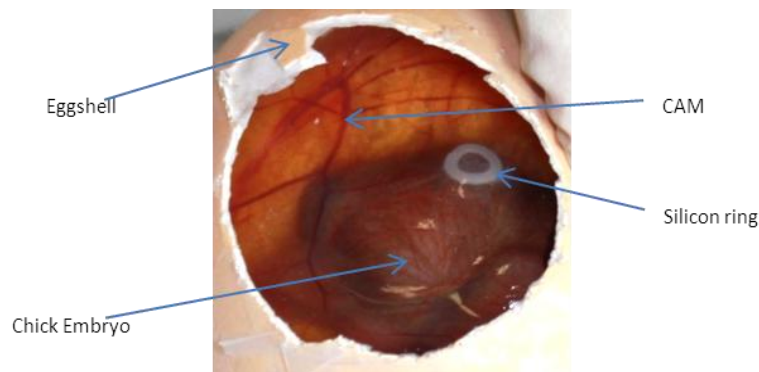


Figure 8 - Timeline regarding CAM Assay procedure.



**Figure 9 - Chick embryo at day 14 of embryonic development.**

### 3.6. Histology



**Figure 10 - Summary of tissue samples processing in paraffin-embedded sections.**

**Processing:** Previously fixed in PFA 4%, tissue samples were dehydrated by increasing concentrations of ethanol (1h at 70%, 1h at 96%, 3h at 100%), cleared with xylene (3h) and infiltrated with paraffin (at 60°C for 2h).

**Embedding:** Processed tissue samples were placed into moulds with liquid paraffin (60°C) and cooled forming hardened blocks.

**Staining:** Haematoxylin and Eosin staining was performed in 3µm paraffin-embedded sections. Deparaffinization and rehydration of all samples was performed with xylene, decreasing concentrations of ethanol (20 minutes at 100%, 5 minutes at 96%, 5 minutes at 70%, 5 minutes at 50%) and water for 5 minutes. Samples were stained with Gill's Haematoxylin III (Thermo Scientific) solution for 5 seconds, differentiated with an ammonia water (0.6%) solution for 15 seconds and with ethanol 95% for 5 minutes and counterstained with Eosin (Thermo Scientific) solution for 5 minutes. Tissue samples were dehydrated with increasing concentrations of ethanol: 95% for 5 minutes, 100% for 10 minutes followed by clear-rite for 10 minutes and embedded with Entellan (CitoCell) mounting medium.

### 3.7. Immunohistochemistry

Paraffin-embedded tissue sections with 3µm were deparaffinised and rehydrated through sequential incubations with xylene (30 minutes), decreasing concentrations of ethanol (100% for 20 minutes; 96% for 10 minutes; 70% for 10 minutes and 50% for 10 minutes) and distilled water.

E-Cadherin antigen retrieval was heat induced with citrate buffer solution (pH 6.0) at 98°C for 30 minutes.

Endogenous peroxidase was blocked with a 3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in methanol solution for 10 minutes, at RT. Unspecific antigens were blocked with Lab Vision Large Volume Ultra V Block (Dako) for 15 minutes, at RT.

Primary antibodies anti-E-Cadherin (1:100, Cell Signalling; and 1:500, BD Biosciences) were diluted in Large Volume Ultra AB Diluent (Dako) and incubated at 4°C overnight. The Envision Detection system (Dako) was used as secondary antibody and incubated at RT for 1 hour. Immunodetection was executed through a peroxidase reaction by adding DAB (110µL/sample) for 3 minutes and counterstained with Gill's Haematoxylin I and dehydration with water (5minutes), increasing concentrations of ethanol (70% for 2 minutes; 96% for 2 minutes; 100% for 4 minutes) and xylene for 5 minutes. Preparations were embedded with Entellan (CitoCell) mounting medium.

### 3.8. Statistical Analysis

For statistical analysis, the unpaired Student's T-test with unequal population size and unequal variance was used to assess the expression phenotype of cells expressing E-Cadherin and cells negative for E-Cadherin regarding E-Cadherin itself, Phospho-ERK1/2 and ERK1/2 (by Western Blot) and *CHD1* and *VEGFA* (by Real-time PCR).

Differences in the number of newly formed blood vessels and in the area of formed micro-tumours regarding E-Cadherin expression within the different cell lines were determined using the same approach with Student's T-test.

*P*-values lower than 0.05 were considered statistically significant.

The dependence between newly formed blood vessels and the area of formed micro-tumours, *in vivo*, was estimated using a linear regression with the Pearson correlation coefficient, *r*.

## Results

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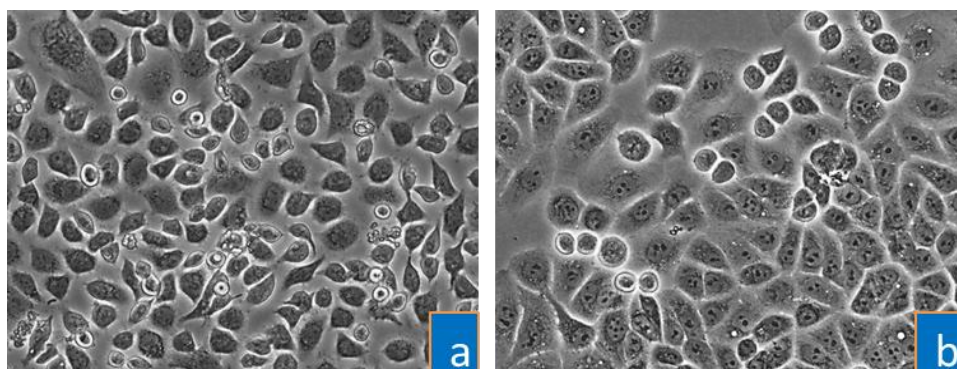
## 4. Results

### 4.1. Characterization of E-Cadherin expressing cell lines

Cell lines stably expressing wild-type (WT) human E-Cadherin or the empty vector have been established as mentioned in the Materials and Methods section. All the constructs have been stably transfected and transduced into mammalian E-Cadherin negative cell lines (AGS and MDA-435) and selected by antibiotic resistance according to the expression vector.

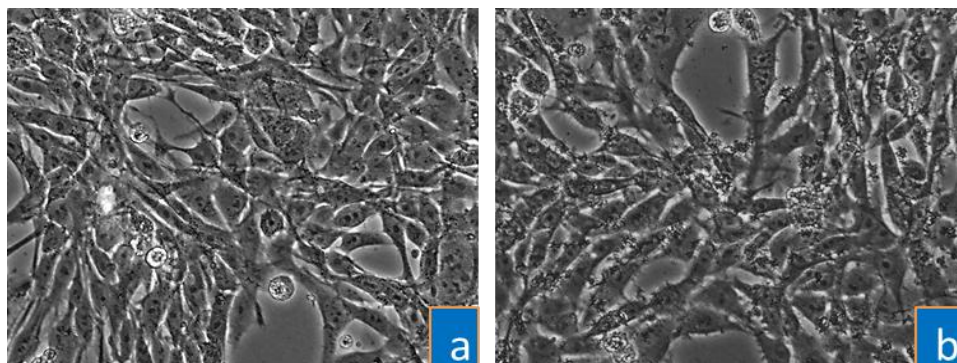
#### 4.1.1. Morphology

Stomach cells, both AGS parental cell line (AGS Par) and cells expressing WT E-Cadherin, AGS WT presented an epithelial morphology with an adherent growth, (Figures 11a and 11b, respectively).



**Figure 11 - Bright field images of AGS Par (a) and WT (b) cells using an Inverted Microscope ECLIPSE TS100-F (Nikon) and a DS Camera Control Unit DS-L2 (Nikon) with 200x of magnification.**

Human breast cell lines, MDA-435 Mock and MDA-435 WT expressing the empty vector and the human WT E-Cadherin, respectively, presented an adherent growth with spindle shaped morphology (Figures 12a and 12b, respectively).



**Figure 12 - Bright field images of MDA-435 Mock (a) and WT (b) cells using an Inverted Microscope ECLIPSE TS100-F (Nikon) and a DS Camera Control Unit DS-L2 (Nikon) with 200x of magnification.**

#### 4.1.2. Protein Expression

To confirm the human WT E-Cadherin expression in the cell lines transfected with the corresponding construct, a western blot analysis to detect E-Cadherin was performed in three or more separate biological replicates. Cells stably expressing WT E-Cadherin showed similar levels of the protein regardless their tissue of origin and cells transfected with the mock vector, E-Cadherin protein was never detected (Figure 13).

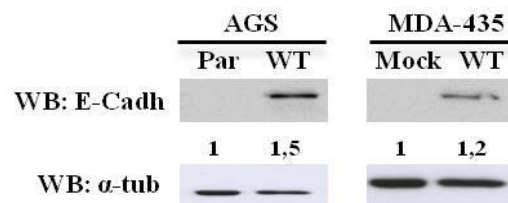


Figure 13 - E-Cadherin expression levels by western blotting analysis of WT human E-Cadherin, normalized for the respective loading control,  $\alpha$ -tubulin: AGS Par and AGS WT (left,  $p=0.02$ ); and MDA-435 Mock and MDA-435 WT (right;  $p=0.12$ ). The values were normalized to the control cells: AGS Par and MDA-435 Mock, respectively.

#### 4.1.3. *CDH1* mRNA transcripts Expression

The levels of *CDH1* mRNA transcripts were determined by Real-time PCR technique in three separate biological replicates, to understand whether E-Cadherin expression was due to the inserted construct or to endogenous expression of the *CDH1* mRNA within the cells. AGS WT showed significantly higher levels of *CDH1* mRNA in comparison to AGS Par ( $p=0.001$ ; Figure 14a). *CDH1* mRNA levels in MDA-435 cells, particularly in MDA-435 WT cells, presented a significant increase in comparison to MDA-435 Mock cells ( $p=0.00005$ ; Figure 14b).

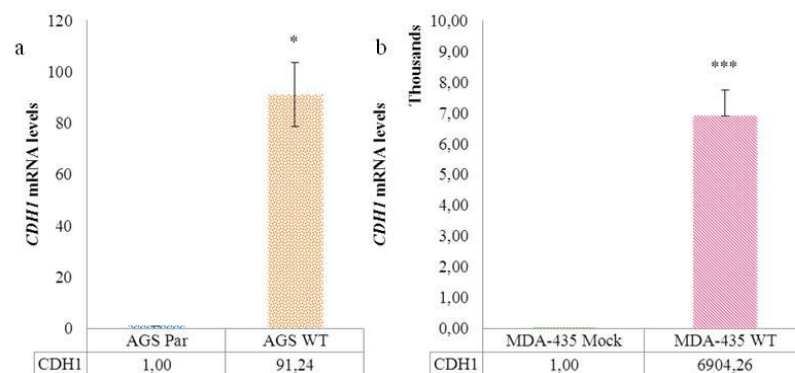


Figure 14 - Relative *CDH1* mRNA expression levels of: a) AGS Par (left) and WT (right) ( $p=0.001$ ); and b) MDA-435 Mock (left) and WT (right) ( $p=0.00005$ ). The values were normalized to the endogenous control expression (*GAPDH*), and to the respective control cell line (AGS Par or MDA-435 Mock).

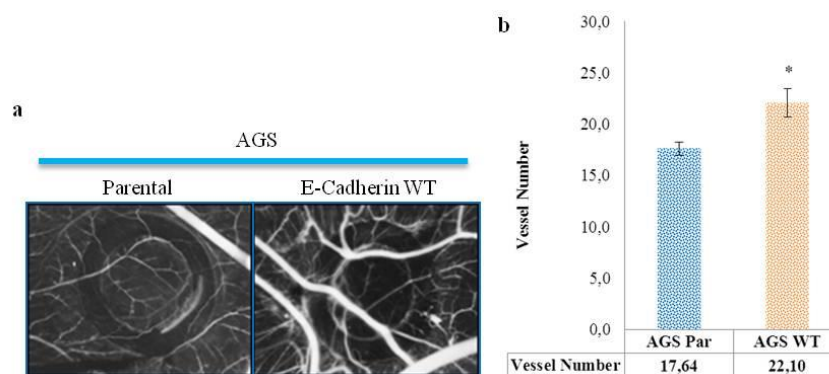
## 4.2. Effects of E-Cadherin on Angiogenesis – Neovascularisation and Micro-tumour formation

To evaluate whether E-Cadherin expression is directly involved in cancer-induced neovascularisation, the CAM assay was performed and both angiogenic and tumorigenic responses were evaluated.

### 4.2.1. E-Cadherin potentiates an angiogenic response

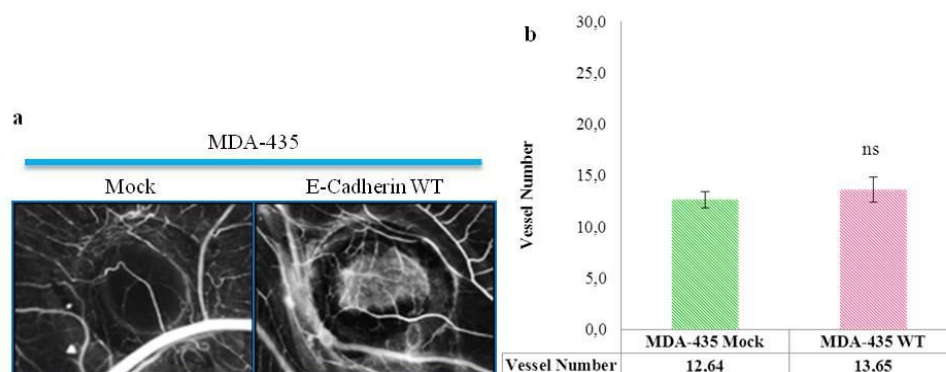
The angiogenic response was evaluated by counting the number of blood vessels with less than 20µm in diameter, growing in a wheel shape towards the inoculation site. The silicone ring delimited a constant area of counting.

AGS cells expressing WT E-Cadherin showed a significant increase ( $p=0.02$ ) in the number of newly formed blood vessels, in comparison to the parental cell line (AGS Par) (Figure 15).



**Figure 15 - Angiogenic response, in the CAM assay, of AGS Par and AGS WT stomach cell lines:** (a) *ex ovo* images of CAM depicting newly formed blood vessels in AGS Par (left) and WT (right); (b) number of newly formed blood vessels in response to AGS Par ( $17.64 \pm 0.65$ ; left) and WT ( $22.10 \pm 1.39$ ; right). AGS WT show a significant increase ( $p=0.02$ ) in the angiogenic response.

For the MDA-435 Mock and WT cell lines the number of newly formed blood vessels were similar ( $p=0.49$ ; Figure 16).

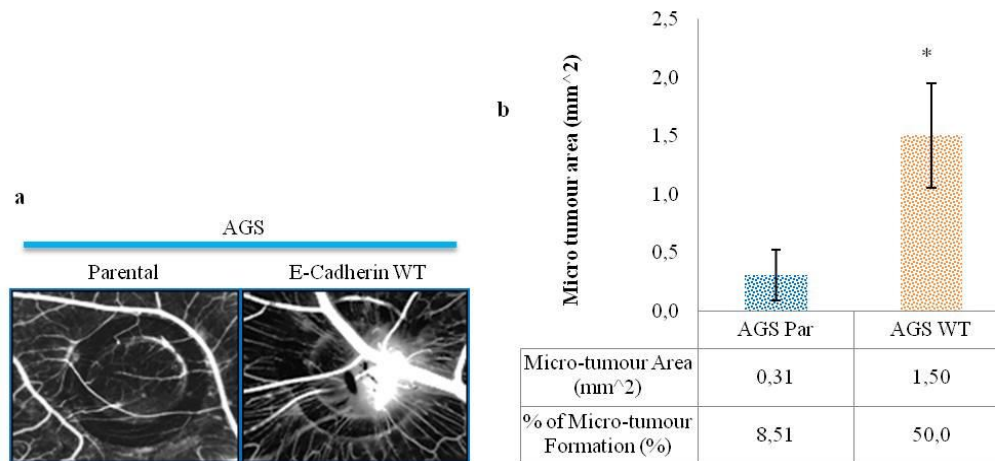


**Figure 16 - Angiogenic response, in the CAM assay, of MDA-435 Mock and MDA-435 WT breast cell lines:** (a) *ex ovo* images of CAM depicting newly formed blood vessels in MDA-435 Mock (left) and WT (right); (b) number of newly formed blood vessels in response to MDA-435 Mock ( $12.64 \pm 0.79$ ; left) and WT ( $13.65 \pm 1.23$ ; right). MDA-435 WT did not show a significant increase ( $p=0.49$ ) in the angiogenic response.

#### 4.2.2. E-Cadherin increases the tumorigenic response

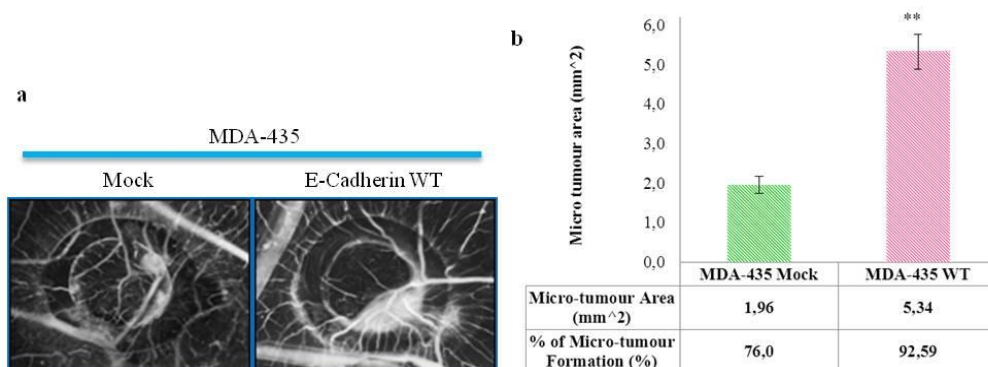
The tumorigenic response was evaluated by assessing the percentage of micro-tumour formation and by measuring micro-tumours areas using Cell A Software (Olympus).

AGS cells expressing WT E-Cadherin showed a statistically significant increase of micro-tumour area ( $p=0.006$ ; Figures 17a and 17b). Moreover, 50% of the cases (15 out of 30 embryos), AGS WT cells formed micro-tumours. AGS Par cells formed micro tumours in 9% of the cases (4 out of 47 embryos).



**Figure 17 - Tumorigenic response in AGS Par and AGS WT stomach cell lines: (a) *ex ovo* images depicting the micro-tumour formation in AGS Par (left) and WT (right); and b) micro-tumours areas of AGS Par ( $0.31 \pm 0.22$ ; left) and WT ( $1.50 \pm 0.45$ ; right) and the percentage of micro-tumour formation in AGS Par (8,5%; left) and WT (50%; right). AGS WT showed significant increase in tumour formation ( $p=0.006$ ).**

MDA-435 WT cells formed significant larger ( $5.34 \pm 0.79$  mm<sup>2</sup>) tumours than MDA-435 Mock cells ( $1.96 \pm 0.42$  mm<sup>2</sup>;  $p=0.0006$ ) (Figure 18b). MDA-435 WT cells formed micro-tumours in 92% of the cases (24 out of 26 embryos) in comparison to MDA-435 Mock cells which formed micro-tumours in 76% of the cases (19 out of 25 embryos).

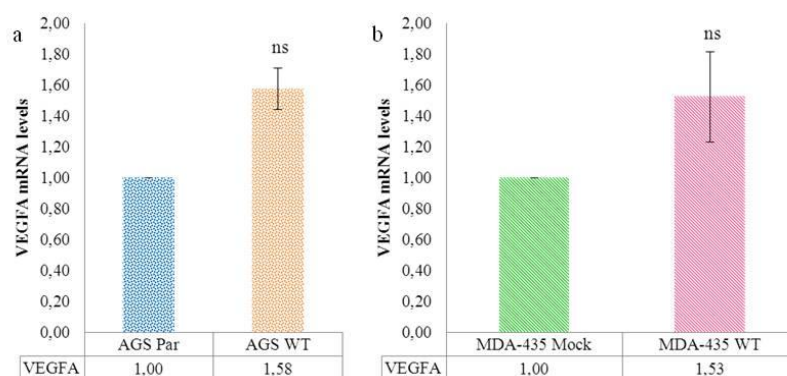


**Figure 18 - Tumorigenic response in MDA-435 Mock and MDA-435 WT breast cell lines: (a) *ex ovo* images depicting the micro-tumour formation in MDA-435 Mock (left) and WT (right); and b) micro-tumours areas of MDA-435 Mock ( $1.96 \pm 0.42$ ; left) and WT ( $5.34 \pm 0.79$ ; right) and the percentage of micro-tumours formation in MDA-435 Mock (76%; left) and WT (92,6%; right). MDA -435 WT showed significant increase in tumour formation ( $p=0.0006$ ).**

No correlation was observed between micro-tumour area and formation in any case.

#### 4.3. *VEGFA* mRNA expression is induced by the presence of E-Cadherin

To evaluate if the expression of Vascular Endothelial Growth Factor – A (VEGF-A) accounts for the functional *in vivo* angiogenic responses of AGS and MDA-435 cell lines, a quantitative Real-time PCR was performed in three separate biological replicates. A two fold increase of *VEGFA* mRNA levels of AGS WT in comparison to AGS Par cells was observed, but without statistical differences ( $p=0.07$ ) (Figure 19a). MDA-435 WT cells presented *VEGFA* mRNA similar to those of MDA-435 Mock ( $p=0.37$ ) (Figure 19b).



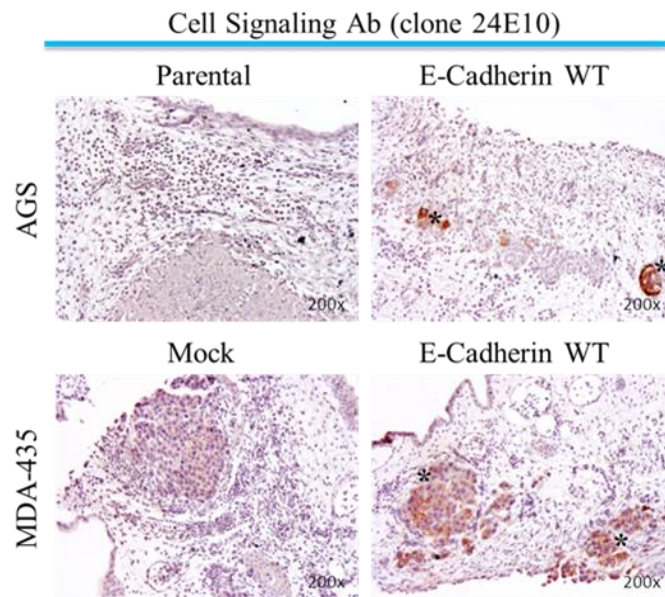
**Figure 19 - Relative *VEGFA* mRNA expression levels within the cell lines: a) AGS Par (left) and WT (right) ( $p=0.07$ ); and b) MDA-435 Mock (left) and WT (right) ( $p=0.37$ ). The values were normalized to the endogenous control, *GAPDH*, expression levels and to the respective control cell lines (AGS Par or MDA-435 Mock).**

#### 4.4. Functional E-Cadherin is being expressed *in vivo*

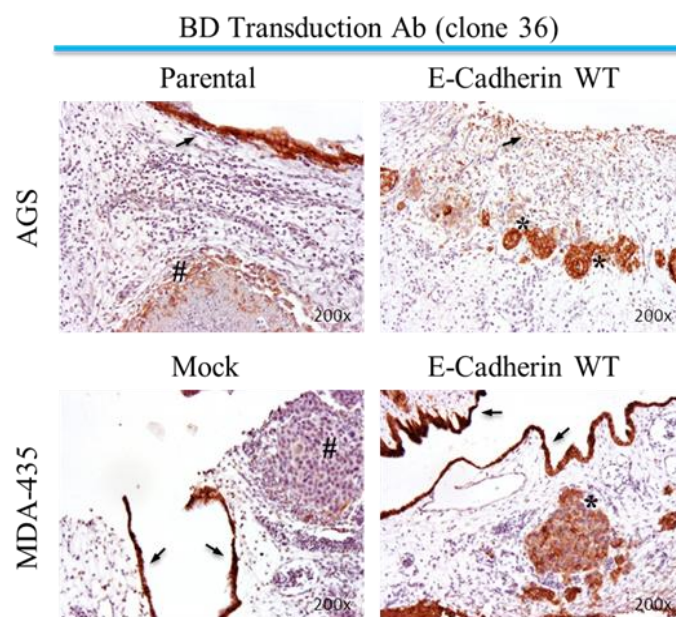
To confirm E-Cadherin expression *in vivo*, an immunostaining was performed in sections of CAM tumours with two different antibodies recognising WT human E-Cadherin (clone 24E10, Cell Signaling; and clone 36, BD Biosciences). The antibodies have different affinities towards E-Cadherin. The antibody 24E10 was specific to human WT E-Cadherin (Figure 20) while the antibody 36 reacted with both human and the E-Cadherin chick endogenous homolog L-CAM (Figure 21).

The results showed a WT E-Cadherin expression, at the cell membrane, only in CAM tumours derived from AGS WT and MDA-435 WT transfected cells, four days after cell inoculation onto CAM (Figure 20). These results confirm human E-Cadherin expression during the angiogenic and tumorigenic assays.





**Figure 20 - Immunohistochemical analysis of WT human E-Cadherin protein (200x of magnification) using a 24E10 antibody (Cell Signaling). Positive immunostaining for E-Cadherin is observed only in CAM tumours derived from WT E-cadherin transfected cells (\*). CAM tumours derived from Mock transfected cells did not express human E-Cadherin.**



**Figure 21 - Immunohistochemical analysis of WT E-Cadherin protein (200x of magnification) using a 36 antibody (BD Biosciences). Positive immunostaining for E-Cadherin is observed in CAM tumours and in CAM epithelial layers (arrows). Labelling intensity of CAM tumours derived from Mock transfected cells (#) is much weaker than that observed in CAM tumours derived from human WT E-cadherin transfected cells (\*).**

#### 4.5. MAPK signalling pathway is activated regardless E-Cadherin expression

MAPKs are ubiquitous intracellular signalling modulators frequently involved in cancer development and progression. To understand if MAPKs mediate the observed *in vivo* angiogenic and tumorigenic responses, the activation and expression profiles of ERK1/2 in all cell lines were assessed by western blot analysis using a phospho-specific antibody in a three or more separate biological replicates.

Although no significant differences in ERK1/2 phosphorylation levels were detected in any of the cell lines (Figure 22), an increase of expression of ERK1/2 phosphorylation was observed in AGS WT (17.2%) and MDA-435 WT (33.6%), in comparison with to AGS Par and MDA-435 Mock, respectively.

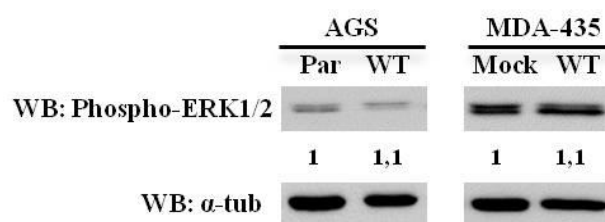


Figure 22 – Phospho-ERK1/2 expression levels by western blotting analysis of phosphorylated ERK1/2, normalized for the respective loading control, α-tubulin: AGS Par and AGS WT (left;  $p=0.74$ ); and MDA-435 Mock and MDA-435 WT (right;  $p=0.61$ ). The values were normalized to the control cells: AGS Par and MDA-435 Mock, respectively.

Immunoblotting for the total forms of this kinase revealed comparable amounts of ERK1/2 MAPK for all the cell lines. There is an increase of expression (4.7%) in AGS WT in comparison with AGS Par (Figure 23). MDA-435 WT cell line showed a decrease of 10.8% in comparison with to MDA-435 Mock (Figures 23).

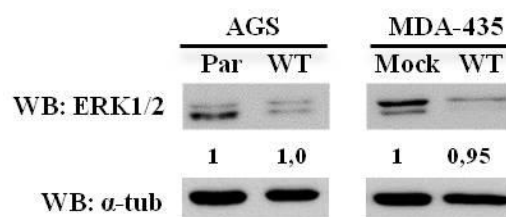


Figure 23 – ERK1/2 expression levels by western blotting analysis of total ERK1/2, normalized for the respective loading control, α-tubulin: AGS Par and AGS WT (left;  $p=0.93$ ); and MDA-435 Mock and MDA-435 WT (right;  $p=0.87$ ). The values were normalized to the control cells: AGS Par and MDA-435 Mock, respectively.





## Discussion and Conclusions

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## 5. Discussion and Conclusions

Cancer is still one of the causes of death worldwide. Despite all the efforts and research, the interaction between some cancer-associated pathways is not yet fully understood.

As an adhesion molecule, E-Cadherin has a pivotal role balancing cell proliferation, adhesion and motility (van Roy and Berx 2008). During tumour initiation and progression, the disruption of E-Cadherin-mediated cell adhesion is a necessary event in the development of epithelial tumours (Guilford 1999). In addition, tumours require a sustained angiogenesis for nutrients and oxygen supply (Hanahan and Folkman 1996). VEGF-A, as an angiogenic regulator, is a signalling protein that binds to stimulatory cell-surface receptors displayed by vascular endothelial cells, maintaining an “angiogenic switch” activated continuously (Hanahan and Folkman 1996; Bergers and Benjamin 2003; Baeriswyl and Christofori 2009; Carmeliet and Jain 2011).

An invasive phenotype is often characterised by E-Cadherin impairment on cell membrane was not yet described to be related with tumour angiogenesis on a molecular level (Hofer and Schweighofer 2007; Bazas, Lukyanova et al. 2008; Zhou, Li et al. 2010). Thus, both *in vitro* and *in vivo* models were needed to understand the role of E-Cadherin expression in tumour angiogenesis.

To analyse and evaluate the relevance of E-Cadherin expression on tumour induced angiogenesis several cell lines were used which presented an epithelial (AGS) or spindle shaped (MDA-435) morphology from different origins: stomach (AGS Par) and breast (MDA-435).

The cell lines were stably transfected with wild-type (WT) human E-Cadherin: AGS WT and MDA-435 WT. All cells expressing WT E-Cadherin presented an organised growth during culturing, suggesting the need of cell-cell contact for “limiting” the uncontrolled growth during tumour development. Such growth was not observed for the control cell lines (AGS Par and MDA-435 Mock).

To confirm that the phenotypical differences observed were indeed related to E-Cadherin presence, transfection efficiency had to be proven both at the mRNA and protein levels. Using quantitative Real-Time PCR on total RNA extracted from different passages of AGS and MDA-435 cell lines it was observed that: 1) MDA-435 WT cells showed significantly higher levels of *CDH1* mRNA whereas MDA-435 Mock completely lacked *CDH1* mRNA ( $p=0.00005$ ); 2) AGS WT cells showed significantly higher levels of *CDH1* mRNA in comparison to AGS Par cells that presented residual *CDH1* transcription ( $p=0.001$ ). The expression of *CDH1* transcripts in parental AGS cells has been previously described and the detected transcripts are known to lead to a truncated form of the protein (Oliveira C, unpublished). To confirm this, Western Blot analysis of total lysates of different cell passages was performed. In fact, AGS Par did not express WT E-Cadherin, similarly to MDA-435

Mock. Moreover, it was observed that all stably WT E-Cadherin transfected cell lines were expressing the cell-cell adhesion protein.

To understand the effect of E-Cadherin introduction into the selected cell lines, in terms of their angiogenic and tumorigenic potential, the chick CAM assay was performed. Tumorigenic potential was first evaluated in terms of micro-tumour area, as a function of size. AGS WT cells showed a significant increase in both angiogenic ( $p=0.02$ ) and tumorigenic ( $p=0.006$ ) responses in comparison to AGS Par cells. MDA-435 WT cells showed a significantly increased tumorigenic response ( $p=0.0006$ ) despite showing no differences in terms of angiogenic potential ( $p=0.49$ ). The assessment of the angiogenic response was difficult to determine due to the morphology of the CAM tumours. These results highlight a role for E-cadherin: its effects on angiogenesis may depend on a specific genetic background of cells.

The tumorigenic potential, via chick CAM assay, can also be analysed in light of micro-tumour formation. AGS WT cells showed micro-tumour formation in 50% of the embryos rather than the 9% of the embryos observed in the absence of E-Cadherin (AGS Par). MDA-435 WT cells also showed an increased percentage of micro-tumour formation (92%) in comparison to the control, MDA-435 Mock (76%). However, no correlation was observed between the size of formed tumours and the percentage of formation.

In conclusion, cells expressing WT E-Cadherin showed a regular outcome concerning the number of newly formed blood vessels and also the occurrence of micro-tumour formation and size. Cells expressing WT E-Cadherin presented always a higher number of newly formed blood vessels, as well as larger and more frequent micro-tumours in comparison with cells lacking E-Cadherin. These results suggest that E-Cadherin might be responsible for cell aggregation within the cells inoculated onto the chick CAM. Thus, it is likely that cell aggregates larger than  $1\text{mm}^2$  in diameter show an increased neovasculature due to their need for nutrients and therefore the need for blood supply (Carmeliet and Jain 2000; McMahon 2000).

To understand whether the angiogenic response observed in the *in vivo* assay was mediated by VEGF-A and if it could be related with E-Cadherin presence, the *VEGFA* mRNA levels were assessed. Using the same approach as previously mentioned for *CDH1*, it was observed that AGS WT and MDA-435 WT, showed increased levels of *VEGFA* mRNA ( $p=0.07$  and  $p=0.37$ , respectively). Not negligible was the presence, although at lower levels, of *VEGFA* transcription in both mock (MDA-435 Mock) and parental (AGS Par) cells, which also displayed micro-tumour formation (76% and 9%, respectively) and a certain degree of neovascularization. The detected transcription of *VEGFA* mRNA within control cells could underlie the observed angiogenic response *in vivo*.

Furthermore, the increased levels of transcription of *VEGFA* mRNA displayed by cells expressing WT E-Cadherin (MDA-435 WT and AGS WT) are in agreement with the *in vivo* functional results, where the angiogenic response was increased in the presence of E-Cadherin. In fact, all tissues and cell types virtually express *VEGFA* mRNA and is particularly elevated in highly vascularised tissue and in tumour-derived cell lines (Berra, Pages et al. 2000; Pages, Milanini et al. 2000). Thus, these results suggest that the presence of functional E-Cadherin might in fact induce an increased expression of *VEGFA* that in turn, mediates the observed increased angiogenic response.

E-Cadherin expression/presence during the *in vivo* assays was determined by immunohistochemistry using two different antibodies recognizing human E-Cadherin. All cell lines expressing WT E-Cadherin (AGS WT and MDA-435 WT) showed E-Cadherin at the plasma membrane in all tested CAM micro-tumours sections. However, the used antibodies showed different affinities towards E-Cadherin: the clone 24E10 (Cell Signaling) was human-specific and reacted only with human E-Cadherin; while the clone 36 (BD Biosciences) was not so specific and reacted also with the chick endogenous homolog, L-CAM, expressed at the CAM. Nevertheless, the clone 36 antibody presented an increased intensity in human E-Cadherin expressing tissue samples (AGS WT and MDA-435 WT), rather than in the controls (AGS Par and MDA-435 Mock). These results confirmed functional E-Cadherin at the plasma membrane during both angiogenic and tumorigenic *in vivo* assays.

It was hypothesized that the increased tumorigenesis and angiogenesis upon E-cadherin presence could be due to MAP kinases signalling. To understand if p42/44 MAPKs (ERK1/2) mediate the observed *in vivo* responses, the activation and expression profiles in all cell lines were assessed by western blot analysis. The results showed similar levels of phospho-ERK1/2 in all cell lines regardless of E-Cadherin expression: AGS WT and MDA-435 WT showed a non-significant increase of expression (17%,  $p=0.74$  and 34%,  $p=0.61$ , respectively). Likewise, total levels of ERK1/2 were similar amongst all cell lines: 1) AGS WT showed a non-significant increase of expression (5%,  $p=0.93$ ); and 2) MDA-435 WT cells showed a non-significant decreased expression (11%,  $p=0.87$ ).

It is known that VEGF-A, as a stimulatory agonist, binds to its receptor expressed on the endothelial surface, VEGFR-2, thus triggering multiple intracellular signalling cascades (Berra, Pages et al. 2000; Breslin, Pappas et al. 2003; Cuenda and Rousseau 2007; Ferrara 2009). Among others, this binding can activate the MAP kinase cascade characterised by the phosphorylation of ERK1/2, a downstream effector (Berra, Milanini et al. 2000; Pages, Milanini et al. 2000; Varma, Breslin et al. 2002; Breslin, Pappas et al. 2003). The observed results showed that E-Cadherin expression increased VEGF-A expression. However, control cells already displayed a basal level of VEGF-A. Therefore, it is possible to assume that such basal level is enough for MAPK pathway activation and that the increased VEGF-A expression due to E-Cadherin presence was not enough to observe an over-activation of this pathway.

The expression of WT E-Cadherin within the cells inoculated onto the chick CAM induced the formation of micro-tumours. Knowing that: 1) larger tumours ( $>1\text{mm}^2$ ) have the need for neovascularisation (Carmeliet and Jain 2000; McMahon 2000); 2) VEGF-A could induce cell motility and proliferation through MAPK signalling pathway (Berra, Milanini et al. 2000; Berra, Pages et al. 2000; Kumar, Shen et al. 2009; Walczak, Gaignier et al. 2011); 3) WT E-Cadherin is being constitutively expressed; and 4) functional E-Cadherin rescue prevents an invasive phenotype (Christofori and Semb 1999; Guilford 1999), it was hypothesised E-cadherin prevention of cell migration lead to larger micro-tumours which needed nutrients and oxygen, hence explaining the increased neovascularisation. However, cells lacking total expression of E-Cadherin also showed neovascularisation and in some cases the formation of micro-tumours. This could be explained by the observed basal levels of *VEGFA* as well as ERK1/2 levels.

In conclusion, in this study it was demonstrated that E-Cadherin is associated with increased levels of VEGF-A. Moreover, we show that E-cadherin in AGS and MDA-435 lead to an increased tumorigenic response. Although both cell lines show distinct neovascularisation responses this response is not dependent on MAPK signalling pathway (ERK1/2). In contrast, the overall results suggest that the angiogenic response to E-Cadherin expression is dependent on the type of cell line in a specific genetic background.

## Future Perspectives

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## 6. Future Perspectives

E-Cadherin is an adhesion molecule expressed in normal epithelial tissues. Its expression in cancer cells derived from epithelia is frequently absent or present abnormal features (Guilford 1999; van Roy and Berx 2008). The ability of cancer cells to migrate and invade to surrounding tissues, implicate cytoskeleton rearrangement (Guilford 1999; van Roy and Berx 2008). External and pro-angiogenic growth factors control these events in a coordinated fashion. In addition, a sustained angiogenesis helps to maintain tumour homeostasis and supply, for example via increased VEGF-A expression.

In this thesis was demonstrated that E-Cadherin may play a direct role in tumour angiogenesis induction. A possible interplay between E-Cadherin and VEGF-A has been identified with E-Cadherin presenting a dual role regarding tissue specificity. On one hand its presence induced higher levels of *VEGFA*, inducing increased neovascularisation in gastric cells. On the other hand, in E-Cadherin's absence, neovascularisation was still observed in breast cells, although at lower levels, in what appears to be a tissue-dependent response. In this regard, some important questions remain unclear:

- Are *in vivo* observations, regarding E-Cadherin expression, being affected by specific cell line programme?
- How can *in vivo* observations differ in a tissue-dependent fashion, regardless of E-Cadherin expression?
- What are the molecules mediating angiogenesis within the context of this study and how do they vary according to E-Cadherin expression?

This could be achieved by pursuing both *in vitro* and *in vivo* studies. A classical approach would be to establish an *in vitro* system with which to manipulate E-Cadherin (by downregulation or by overexpression) aiming at better clarifying its interplay with angiogenesis (VEGF-A expression, MAP Kinase pathway activation, etc).

To complement this approach, E-Cadherin expressing cell lines could be injected in a different *in vivo* model out of the embryonic context, as nude mice. Such model could have simple readouts such as tumour formation and tumour size. In the event of tumour formation in an E-Cadherin dependent manner, immunohistochemical studies could help to understand E-Cadherin impact on tumorigenesis. Another aspect is that VEGF-A expression should provide an insight regarding an angiogenic response related with tumours. Moreover, further investigation on MAP kinase cascades should provide information on *in vivo* cell behaviour regarding cell motility and proliferation, through p38 MAPK activation and the upstream effectors of ERK1/2 pathway.

These and other studies could complement the work presented in this Thesis thus enlightening the role of *CDH1*/E-Cadherin in cancer angiogenesis.

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